Form PTO-1390	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER P21749
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) Not Yet Assigned 09/926661
INTERNATIONAL APPLICATION NO. PCT/JP00/03506	INTERNATIONAL FILING DATE 31 May 2000	PRIORITY DATE CLAIMED 31 May 1999

TITLE OF INVENTION

LYOPHILIZED HGF PREPARATION

APPLICANT(S) FOR DO/EO/US

Masatoshi CHIBA

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. X This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.

2. __ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.

3. X This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).

4. X The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).

5. X A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. X is attached hereto (required only if not communicated by the International Bureau).
   b. X has been communicated by the International Bureau.
   c. __ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. X An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2)).

7. X Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. __ are attached hereto (required only if not communicated by the International Bureau).
   b. __ have been communicated by the International Bureau.
   c. __ have not been made; however, the time limit for making such amendments has NOT expired.
   d. X have not been made and will not be made.

8. __ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3))

9. X An oath or declaration of the inventor(s) (35 U.S.C. .371(c)(4)).
   "UNEXECUTED"

10. __ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (U.S.C. 371(c)(5)).

Items 11 to 16 below concern other document(s) or information included:

11. Assignee: MITSUBISHI CHEMICAL CORPORATION of Ibaraki, JAPAN

12. __ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

13. __ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

14. X A FIRST preliminary amendment.
    __ A SECOND or SUBSEQUENT preliminary amendment.

15. __ A substitute specification.

16. __ A change of power of attorney and/or address letter.

17. __ Figure of Drawing to be published_____

18. X Other items or information:
    Cover Letter
    International Application as published (in Japanese)
    PCT/RO/101 PCT Request (with International Application as filed in Japanese)
    Form PCT/IB/308
    Form PCT/IB/332
    Form PCT/IB/301
    Form PCT/IB/304
    PCT/IPEA/408
    Form PCT/IB/338
    Form PCT/IPEA/409
    Form PCT/ISA/210 (Eng & Jp)
    Claim of Priority

JC13 Rec'd PCT/PTO 29 NOV 2001

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/926661	PCT/JP00/03506	P21749

19. X The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Search report has been prepared by the EPO or JPO. . . . . $ 890.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482). . . . . $ 710.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO(37 CFR 1.445(a)(2). . . . . $ 740.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO. $1,040.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4). . . . . $ 100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$890.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ___ 20 ___ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 0.00	

Claims	Number Filed	Number Extra	RATE		
Total Claims	21 - 20 =	1	X $18.00	$ 18.00	
Independent Claims	4 - 3 =	1	X $84.00	$ 84.00	
Multiple dependent claim(s) (if applicable)			+ $280.00	$ 0.00	
		TOTAL OF ABOVE CALCULATIONS =		$992.00	
Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.				$ 0.00	
		SUBTOTAL =		992.00	
Processing fee of $130.00 for furnishing the English translation later than ___ 20 ___ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				0.00	
Extension of Time fee in the amount of $				0.00	
		TOTAL NATIONAL FEE =		$992.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				0.00	
		TOTAL FEES ENCLOSED =		$992.00	
				Amount to be refunded	$
				Charged	$

a. X A check in the amount of $992.00 to cover the above fees is enclosed.

b. ___ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees.

c. X The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0089.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO **CUSTOMER NO. 7055**
AT THE PRESENT ADDRESS OF:

GREENBLUM & BERNSTEIN, P.L.C.
1941 Roland Clarke Place
Reston, VA 20191
(703) 716-1191

07055
PATENT TRADEMARK OFFICE

Leslie Morgan Kelly
SIGNATURE
Bruce H. Bernstein 33,329
NAME

29,2027
REGISTRATION NUMBER

P21749.A01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Masatoshi CHIBA

Serial No : Not Yet Assigned (National Stage of PCT/JP00/03506)

Filed : Concurrently Herewith (International Filing Date May 31, 2000)

For : LYOPHILIZED HGF PREPARATION

PRELIMINARY AMENDMENT

Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

Prior to calculation of the filing fees and the examination of the above-identified patent application on the merits, the Examiner is respectfully requested to amend the claims as follows:

IN THE CLAIMS

Please amend claims 4-12, 15, 16, 20, and 21 as follows (a marked-up copy of the claim amendments is provided as an attachment to this Amendment):

4. (Amended-Clean Text) The lyophilized preparation according to claim 1, wherein the stabilizing agent is selected from the group consisting of arginine, lysine, histidine, glutamine, proline, glutamic acid, aspartic acid, sulfated polysaccharides, and a pharmacologically acceptable salt thereof.

5. (Amended-Clean Text) The lyophilized preparation according to claim 1, wherein the stabilizing agent is selected from the group consisting of arginine, lysine, histidine, glutamic acid, aspartic acid, and a pharmacologically acceptable salt thereof.

6. (Amended-Clean Text) The lyophilized preparation according to claim 1, wherein the stabilizing agent is selected from the group consisting of arginine, lysine, histidine, and a pharmacologically acceptable salt thereof.

7. (Amended-Clean Text) The lyophilized preparation according to claim 1, wherein the buffering agent is a phosphoric acid salt.

8. (Amended-Clean Text) The lyophilized preparation according to claim 1, wherein the aqueous solution before lyophilization has a pH and an osmotic pressure ratio desirable as an injection.

9. (Amended-Clean Text) The lyophilized preparation according to claim 1, wherein the aqueous solution obtained after redissolution has a pH and an osmotic pressure ratio desirable as an injection.

10. (Amended-Clean Text) The lyophilized preparation according to claim 8, wherein a pH of the aqueous solution before lyophilization is in the range of 5 to 6.5.

11. (Amended-Clean Text) The lyophilized preparation according to claim 8, wherein a pH of the aqueous solution obtained after redissolution is in the range of 5 to 6.5.

12. (Amended-Clean Text) The lyophilized preparation according to claim 1, which further contains a surface active agent.

15. (Amended-Clean Text) The lyophilized preparation according to claim 1, which is prepared in a vial or an ampoule.

16. (Amended-Clean Text) The lyophilized preparation according to claim 1, which contains the stabilizing agent in an amount sufficient to prevent HGF aggregate formation during lyophilization and/or storage after the lyophilization.

20. (Amended-Clean Text) The stabilizing agent according to claim 17, which is selected from the group consisting of arginine, lysine, histidine, glutamic acid, aspartic acid, and a pharmacologically acceptable salt thereof.

21. (Amended-Clean Text) The stabilizing agent according to claim 17, which is selected from the group consisting of arginine, lysine, and a pharmacologically acceptable salt thereof.

REMARKS

By the above amendment, the claims have been amended to delete multiple dependency.

If there should be any questions, the Examiner is invited to contact the undersigned at the telephone number listed below.

Respectfully submitted,
Masatoshi CHIBA

Bruce H. Bernstein
Reg. No. 29,027

33,329

November 27, 2001
GREENBLUM & BERNSTEIN, P.L.C.
1941 Roland Clarke Place
Reston, VA 20191
(703) 716-1191

MARKED-UP COPY OF AMENDED CLAIMS

4. (Amended) The lyophilized preparation according to claim 1 [any one of claims 1 to 3], wherein the stabilizing agent is selected from the group consisting of arginine, lysine, histidine, glutamine, proline, glutamic acid, aspartic acid, sulfated polysaccharides, and a pharmacologically acceptable salt thereof.

5. (Amended) The lyophilized preparation according to claim 1 [any one of claims 1 to 3], wherein the stabilizing agent is selected from the group consisting of arginine, lysine, histidine, glutamic acid, aspartic acid, and a pharmacologically acceptable salt thereof.

6. (Amended) The lyophilized preparation according to claim 1 [any one of claims 1 to 3], wherein the stabilizing agent is selected from the group consisting of arginine, lysine, histidine, and a pharmacologically acceptable salt thereof.

7. (Amended) The lyophilized preparation according to claim 1 [any one of claims 1 to 6], wherein the buffering agent is a phosphoric acid salt.

8. (Amended) The lyophilized preparation according to claim 1 [any one of claims 1 to 7], wherein the aqueous solution before lyophilization has a pH and an osmotic pressure ratio desirable as an injection.

9. (Amended) The lyophilized preparation according to claim 1 [any one of claims 1 to 7], wherein the aqueous solution obtained after redissolution has a pH and an osmotic pressure ratio desirable as an injection.

10. (Amended) The lyophilized preparation according to claim 8 [or 9], wherein a pH of the aqueous solution before lyophilization is in the range of 5 to 6.5.

11. (Amended) The lyophilized preparation according to claim 8 [or 9], wherein a pH of the aqueous solution obtained after redissolution is in the range of 5 to 6.5.

12. (Amended) The lyophilized preparation according to claim 1 [any one of claims 1 to 11], which further contains a surface active agent.

15. (Amended) The lyophilized preparation according to claim 1 [any one of claims 1 to 14], which is prepared in a vial or an ampoule.

16. (Amended) The lyophilized preparation according to claim 1 [any one of claims 1 to 15], which contains the stabilizing agent in an amount sufficient to prevent HGF aggregate formation during lyophilization and/or storage after the lyophilization.

20. (Amended) The stabilizing agent according to claim 17 [any one of claims 17 to 19], which is selected from the group consisting of arginine, lysine, histidine, glutamic acid, aspartic acid, and a pharmacologically acceptable salt thereof.

21. (Amended) The stabilizing agent according to claim 17 [any one of claims 17 to 19], which is selected from the group consisting of arginine, lysine, and a pharmacologically acceptable salt thereof.

P21749.P02

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Masatoshi CHIBA

Serial No. : Not Yet Assigned

Filed : Concurrently Herewith

For : LYOPHILIZED HGF PREPARATION

CLAIM OF PRIORITY

Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

Applicant hereby claims the right of priority granted pursuant to 35 U.S.C. 119 based upon Japanese Application No. 11-151769, filed May 31, 1999. The International Bureau already should have sent a certified copy of the Japanese application to the United States deesignated office. If the certified copy has not arrived, please contact the undersigned.

Respectfully submitted,
Masatoshi CHIBA

Bruce H. Bernstein
Reg. No. 29,027

Reg. No. 33,329

November 28, 2001
GREENBLUM & BERNSTEIN, P.L.C.
1941 Roland Clarke Place
Reston, VA 20191
(703) 716-1191

SPECIFICATION

Lyophilized HGF Preparation

Technical Field

The present invention relates to a lyophilized preparation comprising a hepatocyte growth factor.

Background Art

Hepatocyte growth factor (abbreviated occasionally as "HGF" hereafter in the specification) is a protein having a proliferating activity of hepatocytes and its existence in various animal species is known. HGFs having different amino acid sequences have been reported. Human hepatocyte growth factor (abbreviated occasionally as "hHGF" hereafter in the specification) was found from plasma of a fulminant hepatitis patient by Daikuhara et al. (Japanese Patent Unexamined Publication (Kokai) No. 63-22526). The amino acid sequence of the hHGF protein and the gene (cDNA) sequence encoding said protein were found by Kitamura et al. (Japanese Patent Unexamined Publication No. 3-72883). A method for producing the hHGF protein and a transformant using said cDNA have been reported (Japanese Patent Unexamined Publication No. 3-285693). Under the circumstances, mass production of the hHGF protein becomes possible and its application as a medicament is expected.

hHGF is a kind of glycoprotein, which is a heterodimer consisting of α subunit having a molecular weight of about 80-90 kDa in a non-reduced state or about 52-56 kDa in a reduced state and β subunit having a molecular weight of about 30-36 kDa. Besides the activity as hepatic cell growth factor, hHGF has various biological activities such as a scatter factor (SF) activity, renal tubular epithelial cell growth factor activity, damaged tissue repair factor activity and vascular endothelial cell growth factor activity, and the protein is expected to be developed as medicaments for therapeutic treatment of liver diseases, kidney diseases, cranial nerve disorders, hair growth promoters, wound healing agents, antitumor therapeutic agents and the like.

Pharmaceutical preparations of HGF are described in WO90/10651 and Japanese Patent Unexamined Publication Nos. 6-247872 and 9-25241. The

aforementioned WO90/10651 discloses an aqueous preparation of deletion-type HGF (TCF) in which five amino acid residues are deleted from HGF, and the publication teaches that albumin, human serum, gelatin, sorbitol, mannitol, xylitol and the like stabilize TCF in an aqueous solution. Japanese Patent Unexamined Publication No. 6-247872 discloses an injection containing TCF at a high concentration of 5-10 mg/mL in which a basic amino acid or the like coexists with TCF. This publication refers to the solubility of TCF in an aqueous solution and discloses an aqueous solution containing TCF at a high concentration. The basic amino acid (lysine, arginine) is used as a "solubilizing aid" in the injection.

However, the aqueous HGF preparation rapidly decreases the solubility of HGF at a neutral pH and has a problem of progress of aggregation, cloudiness and gelation when stored at a low temperature or room temperature for several days. Further, the preparation has low physicochemical stability, for example, formation of degradation products and aggregates, and also has poor stability as a pharmaceutical preparation, for example, reduce of biological activity. Therefore, the preparation is not suitable for a long-term storage from a viewpoint of biological activity. Furthermore, the aqueous HGF preparation may cause aggregation, cloudiness, and gelation due to foaming or the like after shaking and stirring, which leads to decreases of quality of a pharmaceutical preparation and drug efficacy during long-term storage, distribution and transportation. Therefore, a lyophilized preparation is preferred as an HGF preparation.

Japanese Patent Unexamined Publication No. 9-25241 discloses a lyophilized preparation of HGF (TCF). However, unlike the present invention, the patent publication teaches that a lyophilized preparation comprising HGF (TCF) at a high concentration that is stable over a long period can be provided by using a citrate as a buffering agent and glycine, alanine, sorbitol, mannitol or the like as a stabilizing agent. However, due to the citric acid used as a buffering agent in the lyophilized preparation, a pH of a redissolved preparation will be in an acidic condition. Further, the resulting solution has a high osmotic pressure, which causes problems of pain at administration by injection, or inflammatory reaction and hemolysis at an administration site and the like.

HGF is a substance having extremely potent physiological activities, and when used as a medicament, the substance needs to be provided in the clinical filed as a

pharmaceutical preparation having a very low concentration. Studies by the inventors of the present invention revealed that, as for the lyophilized HGF (TCF) preparation comprising glycine or alanine described in Japanese Patent Unexamined Publication No. 9-25241, only a little formation of aggregates was observed during storage when the lyophilized preparation was produced from an aqueous solution containing HGF at a high concentration, whilst aggregate formation was observed during storage when a preparation was produced in the presence of glycine or alanine by lyophilizing an aqueous solution containing HGF at a low concentration, which is desirable for clinical application (generally, HGF is contained at a concentration lower than 5 mg/mL, for example, about 2 mg/mL). Accordingly, glycine or alanine described in Japanese Patent Unexamined Publication No. 9-25241 is useful as a stabilizing agent when HGF is lyophilized at a high concentration, however, the amino acid is not sufficient as a stabilizing agent when HGF is lyophilized at a low concentration. It has therefore been desired to develop a method for producing a lyophilized preparation that hardly forms aggregates and has excellent stability in long-term storage by using an aqueous solution containing HGF at a low concentration.

Disclosure of the Invention

An object of the present invention is to provide a lyophilized HGF preparation that can produce an aqueous solution containing HGF at a low concentration. More specifically, the object of the present invention is to provide a lyophilized HGF preparation that has excellent storage stability and is free from aggregation, cloudiness, gelation or the like upon redissolution. Another object of the present invention is to provide a lyophilized preparation that has a favorable cake forming property during lyophilization and excellent re-solubility. Yet another object of the present invention is to provide the preparation having a pH and an osmotic pressure ratio desirable as an injection.

The inventors of the present invention conducted various studies to achieve the foregoing objects. As a result, they found that, when a solution containing HGF at a concentration lower than 5 mg/mL was lyophilized in the presence of a stabilizing agent, sodium chloride and a buffering agent, a lyophilized preparation having a favorable cake forming property, solubility and long-term storage stability was

successfully produced, and that aggregates were not formed in the production of the lyophilized preparation as well as during storage of said preparation, and the lyophilized preparation had extremely high stability. Further, they also found that, an aqueous solution prepared from the lyophilized preparation was free from aggregation, cloudiness, gelation or the like, and the aqueous solution containing the low concentration of HGF successfully exert sufficient clinical effectiveness. The present invention was achieved on the basis of the above findings.

The present invention thus provides:

a lyophilized preparation comprising a hepatocyte growth factor, a stabilizing agent for preventing formation of a aggregate of the hepatocyte growth factor, sodium chloride and a buffering agent, which is prepared from an aqueous solution containing the hepatocyte growth factor at a concentration lower than 5 mg/mL;

a lyophilized preparation comprising a hepatocyte growth factor, a stabilizing agent for preventing formation of a aggregate of the hepatocyte growth factor, sodium chloride and a buffering agent, which is for preparing after redissolution an aqueous solution containing the hepatocyte growth factor at a concentration lower than 5 mg/mL; and

a lyophilized preparation comprising a hepatocyte growth factor, a stabilizing agent for preventing formation of a aggregate of the hepatocyte growth factor, sodium chloride and a buffering agent, which is prepared from an aqueous solution containing the hepatocyte growth factor at a concentration lower than 5 mg/mL, and which is for preparing after redissolution an aqueous solution containing the hepatocyte growth factor at a concentration lower than 5 mg/mL.

These lyophilized HGF preparations do not give HGF aggregates during lyophilization and long-term storage after the lyophilization and have excellent stability. Further, they have characteristic features that aggregation, cloudiness, gelation or the like does not occur in an aqueous solution prepared from the lyophilized preparation, and, even after storage of the aqueous solution, aggregates are hardly formed.

Usually, it is preferred that an aqueous solution used to prepare a lyophilized preparation in a vial and an aqueous solution prepared by dissolving the resulting lyophilized preparation in the vial contain the same concentration of an active ingredient. Therefore, the preparation of the present invention can preferably be

produced by lyophilizing an aqueous solution containing HGF at a concentration lower than 5 mg/mL in a vial or an ampoule. Further, as for the preparation of the present invention, a pH of the aqueous solution before lyophilization and/or the aqueous solution obtained after redissolution is preferably in the range of 5 to 6.5, which is desirable as an injection. Also as for the preparation of the present invention, the aqueous solution before lyophilization and/or the aqueous solution obtained after redissolution preferably have an osmotic pressure desirable as an injection, for example, almost isotonic in living bodies or osmotic pressure ratio acceptable as an injection (1 to 2).

According to preferred embodiments of the present invention, there are provided the aforementioned lyophilized HGF preparation, wherein the stabilizing agent is selected from the group consisting of arginine, lysine, histidine, glutamine, proline, glutamic acid, aspartic acid, sulfated polysaccharides and pharmacologically acceptable salts thereof; the aforementioned lyophilized HGF preparation, wherein the stabilizing agent is selected from the group consisting of arginine, lysine, histidine, glutamic acid, aspartic acid and pharmacologically acceptable salts thereof; the aforementioned lyophilized HGF preparation, wherein the stabilizing agent is selected from the group consisting of arginine, lysine, histidine and pharmacologically acceptable salts thereof; and the aforementioned lyophilized HGF preparation, wherein the stabilizing agent is selected from the group consisting of arginine, lysine and pharmacologically acceptable salts thereof. These stabilizing agents are preferably added to the preparation in an amount sufficient to prevent formation of a aggregate of HGF during lyophilization and/or storage after the lyophilization.

Further, according to other preferred embodiments of the present invention, there are provided the aforementioned lyophilized HGF preparation, wherein the buffering agent is a phosphoric acid salt; the aforementioned lyophilized HGF preparation, which further contains a surface active agent; the aforementioned lyophilized HGF preparation, wherein the surface active agent is a nonionic surface active agent; and the aforementioned lyophilized preparation, wherein the nonionic surface active agent is a polyoxyethylene ether surface active agent.

From another aspect of the present invention, there is provided a stabilizing agent for HGF used to lyophilize an aqueous solution containing HGF at a concentration lower than 5 mg/mL, which is selected from the group consisting of

arginine, lysine, histidine, glutamine, proline, glutamic acid, aspartic acid, sulfated polysaccharides, and pharmacologically acceptable salts thereof. Preferred stabilizing agents are selected from the group consisting of arginine, lysine, histidine, glutamic acid, aspartic acid, and pharmacologically acceptable salts thereof. Particularly preferred stabilizing agents are selected from the group consisting of arginine, lysine, and pharmacologically acceptable salts thereof. These stabilizing agents can prevent formation of a aggregate of HGF during lyophilization of an aqueous solution containing HGF at a concentration of lower than 5 mg/mL and during storage after the lyophilization.

The present invention further provides a lyophilized HGF preparation, which can be obtained by lyophilizing an aqueous solution containing the aforementioned stabilizing agent and HGF at a concentration lower than 5 mg/mL and is used to prepare an aqueous solution containing HGF at a concentration lower than 5 mg/mL by redissolution. A preferred preparation of the aforementioned lyophilized preparation can be obtained by lyophilizing an aqueous solution containing the stabilizing agent (preferably, a stabilizing agent selected from a group consisting of arginine, lysine and pharmacologically acceptable salts thereof), HGF at a concentration lower than 5 mg/mL, sodium chloride and a buffering agent in a vial.

Best Mode for Carrying out the Invention

The type of HGF contained in the lyophilized preparation of the present invention is not particularly limited. For example, natural HGF may be isolated from humors or tissues derived from mammals such as human and rat, which are known to contain HGF, or cells that spontaneously produce HGF. A recombinant HGF obtained by introducing cDNA of said growth factor into cells by gene recombination technique may also be used. Examples of hosts for producing a recombinant HGF include *Escherichia coli*, *Bacillus subtilis*, yeast, filamentous fungi, plant cells, insect cells, animal cells and the like. Specific examples of the recombinant HGF include those obtained from placenta derived from the mammals, liver tissues and blood of a hepatopathy patient, fibroblast strains such as MRC-5 cells and IMR-9 cells, strains producing HGF obtained by introducing an expression vector including cDNA encoding hHGF into a host such as CHO cells according to the method described in Japanese Patent Unexamined Publication No. 3-285693 and the like.

Further, as HGF, a precursor protein such as a protein having a signal sequence, a modified protein wherein some of amino acids are replaced, deleted and/or inserted so as not to deteriorate the activity of proliferating hepatocytes, or an altered protein wherein a saccharide is deleted or replaced. Examples of the altered protein include those described in Japanese Patent Unexamined Publication No. 2-288899, WO90/10651, Japanese Patent Unexamined Publication Nos. 3-130091, 3-255096 and 4-30000, Nature, 342, pp.440-443 (1989) and the like.

Examples of HGF preferably used for the lyophilized preparation of the present invention include proteinic factors having the following physicochemical properties. The HGF is preferably derived from human. Examples of particularly preferred HGF include those having the amino acid sequences described in Japanese Patent Unexamined Publication Nos. 3-72883 and 4-89499.

1) The factor has an estimated molecular weight of about 76,000-92,000 by SDS-PAGE (under non-reducing condition);

2) the factor has the activity of proliferating hepatocytes; and

3) the factor has strong affinity for heparin.

Further, in addition to the above physicochemical properties, preferred HGF has the following properties:

4) The aforementioned activities are inactivated by a heat treatment at 80°C for 10 minutes; and

5) the aforementioned activities are inactivated by digestion with trypsin or chymotrypsin.

Lyophilized HGF preparations containing three ingredients of HGF, a buffering agent and sodium chloride (those described in Japanese Patent Unexamined Publication Nos. 6-247872 and 9-25241: HGF concentration is 5-20 mg/mL) have a problem that, when the content of HGF is reduced to avoid problems such as precipitation of HGF, a favorable cake cannot be obtained in lyophilization process. Further, there is also a problem that aggregation, cloudiness and gelation are observed in an aqueous solution obtained by redissolving a lyophilized preparation obtained from the above three ingredients, and thus sufficient physicochemical stability cannot be attained. Therefore, to prepare a lyophilized preparation that can give a favorable cake form by lyophilization and enables production of an aqueous solution having excellent long-term storage stability, it is essential to add an additive to improve a

cake forming property and storage stability in the state of an aqueous solution.

The lyophilized preparation of the present invention is manufactured from an aqueous solution containing HGF at a concentration lower than 5 mg/mL and/or prepared so that an aqueous solution produced from the lyophilized preparation contains HGF at a concentration lower than 5 mg/mL. Preferably, the lyophilized preparation can be manufactured so that an aqueous solution before lyophilization and/or an aqueous solution obtained after redissolution have a pH desirable as an injection and have substantial isotonicity with living bodies or an osmotic pressure ratio acceptable as an injection (1 to 2). The lyophilized preparation of the present invention is characterized to have excellent storage stability. The lyophilized preparation is also characterized in that the preparation can form a favorable lyophilization cake in lyophilization process, and that an aqueous solution obtained by redissolving the lyophilized preparation is free from a problem of aggregation, cloudiness or gelation, thereby sufficient physicochemical stability is achieved. Furthermore, in clinical applications, the preparation can sufficiently exert desired pharmacological actions.

Examples of the stabilizing agent include arginine, lysine, histidine, glutamine, proline, glutamic acid, aspartic acid, sulfated polysaccharides such as heparin, chondroitin sulfate, dermatan sulfate, heparan sulfate, keratan sulfate, and dextran sulfate, and pharmacologically acceptable salts thereof. Examples of the pharmacologically acceptable salts include alkali metal salts such as sodium salts and potassium salts. These stabilizing agents may be used as a combination of two or more kinds. Examples of preferred stabilizing agents include arginine, lysine, histidine, glutamic acid, aspartic acid and the like. Among them, arginine, lysine, histidine and a combination thereof are particularly preferred. The amount of the stabilizing agent to be added is not particularly limited as long as the storage stability of HGF can be achieved, but is preferably 0.01-100 times by weight, most preferably 0.1-30 times by weight based on the weight of HGF.

The buffering agent is not also particularly limited so long as the agent has an action for adjusting pH of the aqueous solutions before lyophilization and after redissolution and maintaining solubility of HGF. For example, a phosphate buffer, a citrate buffer, an acetate buffer or the like can be used. As the buffering agent, a phosphate buffer, particularly preferably, a sodium phosphate buffer can be preferably

used. The amount of the buffering agent to be added is, for example, about 1-100 mM based on the amount of water after redissolution.

Sodium chloride improves the solubility of HGF in the aqueous solutions before lyophilization and after redissolution, however, it is not preferred to add sodium chloride more than necessary, because it increases osmotic pressure. In general, it is sufficient to add sodium chloride in an amount sufficient to achieve an isotonic osmotic pressure with living bodies. The osmotic pressure ratio is most preferably 1-2, which is acceptable as the osmotic pressure ratio of an injection. For example, it is preferable to add 140 mM of sodium chloride based on the volume of water after redissolution.

HGF has a problem that its solubility is rapidly decreased at neutral pH, since the pH overlaps with the isoelectric point of HGF (pI = 7-8). For example, HGF has low solubility of a little less than 1.0 mg/mL around pH 7.0-7.5 in 10 mM sodium phosphate buffer (PBS, room temperature) containing 140 mM sodium chloride. Whilst HGF has a solubility of 5 mg/mL or higher around pH 5.0, and the solubility of HGF becomes higher at a lower pH. Further, at a sodium chloride concentration of 0.14 M, the solubility of HGF is about 1 mg/mL, and when the concentration is made 0.3 M or higher, HGF is dissolved at a concentration of 5 mg/mL or higher. Therefore, it is also conceived that, to increase the solubility of HGF, the solution is kept in an acidic condition at a pH of 5 or lower or the sodium chloride concentration is increased to 0.3 M or higher. In the preparation of the present invention, it is preferred that pH of the aqueous solutions before lyophilization and/or after redissolution is adjusted to be within a weakly acidic range, specifically at a pH of 4.0-6.5, preferably a pH of 5.0-6.5. In such a pH range, formation of a aggregate is suppressed.

The lyophilized HGF preparation of the present invention is preferably added further with a surface active agent. HGF is easily adsorbed to a container material such as glass or a resin. At a low concentration, in particular, adsorption of HGF to a container leads to decrease of a drug content in a solution to be administered. By adding a surface active agent, adsorption of HGF to a container after redissolution can be prevented. Examples of the surface active agent include nonionic surface active agents such as Polysorbate 80, Polysorbate 20, HCO-40, HCO-60, Pluronic F-68 and polyethylene glycol, and a combination of two or more kinds of these agents may also be used. As the surface active agent, polyoxyethylene ether surface active agents

(Polysorbate 80 and the like) can be most preferably used. The amount of the surface active agent is, for example, in a range of 0.001-2.0% by weight based on the weight of water after redissolution.

The lyophilized HGF preparation of the present invention can be produced by lyophilizing an aqueous solution containing HGF according to a conventional method. For example, HGF, a stabilizing agent, sodium chloride and a buffering agent can be dissolved in distilled water for injection, optionally added with a surface active agent, sterilized by filtration and introduced into a container such as a vial or an ampoule, and then subjected to lyophilization. The lyophilized HGF preparation of the present invention may contain other additives necessary for formulation, for example, antioxidants, preservatives, excipients, soothing agents and the like. An example of the lyophilization method includes, for example, a method comprising three unit operations: (1) a freezing step for chilling and freezing under atmospheric pressure, (2) a primary drying step for sublimating and drying free water not restrained by a solute under reduced pressure, and (3) a secondary drying step for removing adsorbed water or crystal water intrinsic to the solute (Pharm. Tech. Japan, 8 (1), pp.75-87, 1992). However, the method for producing the lyophilized preparation of the present invention is not limited to the above method. The lyophilized preparation of the present invention can be dissolved by adding a solvent such as distilled water for injection upon use so that the HGF concentration becomes lower than 5 mg/mL.

Examples

The present invention will be explained more specifically with reference to the following examples. However, the scope of the present invention is not limited to these examples.

Example 1: Preparation of a lyophilized low-concentration HGF preparation (Comparative Example)

HGF is dissolved at a concentration of 1 mg/mL in 10 mM phosphate buffer (pH 6.5) containing 140 mM sodium chloride and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution is adjusted, the solution is aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation can be

obtained by lyophilizing the solution according to the conditions shown in Table 1. In the table, "→" indicates that temperature is changed.

Table 1

	Freezing process		Primary drying process		Secondary drying process	
Temperature (°C)	20 → -40	-40	-40 → -20	-20	-20 → 20	20
Time (Hr)	1	5	3	48	2	24
Pressure (mmHg)	760	760	< 1	< 1	< 1	< 1

Example 2: Preparation of a lyophilized low-concentration HGF preparation (Comparative Example)

HGF is dissolved with heating at a concentration of 5 mg/mL in 10 mM phosphate buffer (pH 6.5) containing 140 mM sodium chloride and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution is adjusted, the solution is aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation can be obtained by lyophilizing the solution according to the conditions shown in Table 1.

Example 3: Preparation of a lyophilized high-concentration HGF preparation (Comparative Example)

HGF is dissolved at a concentration of 10 mg/mL in 10 mM phosphate buffer (pH 6.5) containing 140 mM sodium chloride, 100 mM arginine and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution is adjusted, the solution is aseptically charged into vials in an amount of 2 mL per vial. A lyophilized high-concentration HGF preparation can be obtained by lyophilizing the solution according to the conditions shown in Table 1.

Example 4: Preparation of a lyophilized low-concentration HGF preparation (Comparative Example)

HGF is dissolved at a concentration of 1 mg/mL in 10 mM citrate buffer (pH 5.0) containing 300 mM sodium chloride and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution is adjusted, the solution is aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation can be obtained by lyophilizing the solution according to the conditions shown in Table 1.

Example 5: Preparation of a lyophilized low-concentration HGF preparation (Comparative Example)

HGF is dissolved at a concentration of 1 mg/mL in 10 mM citrate buffer (pH 5.0) containing 300 mM sodium chloride, 5% glycine and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution is adjusted, the solution is aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation can be obtained by lyophilizing the solution according to the conditions shown in Table 1.

Example 6: Preparation of a lyophilized low-concentration HGF preparation (Comparative Example)

HGF is dissolved at a concentration of 1 mg/mL in 10 mM citrate buffer (pH 5.0) containing 300 mM sodium chloride, 5% alanine and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution is adjusted, the solution is aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation can be obtained by lyophilizing the solution according to the conditions shown in Table 1.

Example 7: Preparation of a lyophilized low-concentration preparation (Present Invention)

HGF is dissolved at a concentration of 1 mg/mL in 10 mM phosphate buffer (pH 6.5) containing 140 mM sodium chloride and 100 mM arginine and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the

aqueous solution is adjusted, the solution is aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation can be obtained by lyophilizing the solution according to the same conditions as in Example 1. Upon use, this preparation can be dissolved in 2 mL of distilled water for injection to obtain an injection containing HGF at a concentration of 1 mg/mL and having a pH and osmotic pressure ratio (1.5, almost isotonic) acceptable as an injection.

Example 8: Preparation of a lyophilized low-concentration preparation (Present Invention)

HGF is dissolved at a concentration of 1 mg/mL in 10 mM phosphate buffer (pH 6.5) containing 140 mM sodium chloride, 100 mM arginine and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution is adjusted, the solution is aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation can be obtained by lyophilizing the solution according to the same conditions as in Example 1.

Example 9: Preparation of a lyophilized low-concentration preparation (Present Invention)

HGF is dissolved at a concentration of 2 mg/mL in 10 mM phosphate buffer (pH 6.5) containing 140 mM sodium chloride, 100 mM arginine and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution is adjusted, the solution is aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation can be obtained by lyophilizing the solution according to the same conditions as in Example 1.

Example 10: Preparation of a lyophilized low-concentration preparation (Present Invention)

HGF is dissolved at a concentration of 3 mg/mL in 10 mM phosphate buffer (pH 6.5) containing 140 mM sodium chloride, 100 mM arginine and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution is adjusted, the solution is aseptically charged into

vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation can be obtained by lyophilizing the solution according to the same conditions as in Example 1.

Example 11: Preparation of a lyophilized low-concentration preparation (Present Invention)

HGF is dissolved at a concentration of 4 mg/mL in 10 mM phosphate buffer (pH 6.5) containing 140 mM sodium chloride, 100 mM arginine and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution is adjusted, the solution is aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation can be obtained by lyophilizing the solution according to the same conditions as in Example 1.

Example 12: Preparation of a lyophilized low-concentration preparation (Comparative Example)

HGF is dissolved at a concentration of 5 mg/mL in 10 mM phosphate buffer (pH 6.5) containing 140 mM sodium chloride, 100 mM arginine and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution is adjusted, the solution is aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation can be obtained by lyophilizing the solution according to the same conditions as in Example 1.

Example 13: Preparation of a lyophilized low-concentration preparation (Present Invention)

A lyophilized low-concentration HGF preparation can be obtained by dissolving HGF at a concentration of 1 mg/mL in the same manner as in Example 8 by using 10 mM phosphate buffer (pH 6.0) instead of 10 mM phosphate buffer (pH 6.5).

Example 14: Preparation of a lyophilized low-concentration preparation (Present Invention)

A lyophilized low-concentration HGF preparation can be obtained by

dissolving HGF at a concentration of 1 mg/mL in the same manner as in Example 8 by using 10 mM phosphate buffer (pH 5.5) instead of 10 mM phosphate buffer (pH 6.5).

Example 15: Preparation of a lyophilized low-concentration preparation (Present Invention)

A lyophilized low-concentration HGF preparation can be obtained by dissolving HGF at a concentration of 1 mg/mL in the same manner as in Example 8 by using 10 mM phosphate buffer (pH 5.0) instead of 10 mM phosphate buffer (pH 6.5).

Example 16: Preparation of a lyophilized low-concentration preparation (Present Invention)

A lyophilized low-concentration HGF preparation can be obtained by dissolving HGF at a concentration of 1 mg/mL in the same manner as in Example 8 by using 10 mM phosphate buffer (pH 7.2) instead of 10 mM phosphate buffer (pH 6.5).

Example 17: Preparation of a lyophilized low-concentration preparation (Present Invention)

A lyophilized low-concentration HGF preparation can be obtained by dissolving HGF at a concentration of 1 mg/mL in the same manner as in Example 8 by using 10 mM phosphate buffer (pH 7.0) instead of 10 mM phosphate buffer (pH 6.5).

Example 18: Preparation of a lyophilized low-concentration preparation (Present Invention)

A lyophilized low-concentration HGF preparation can be obtained in the same manner as in Example 8 by using 50 mM arginine instead of 100 mM arginine.

Example 19: Preparation of a lyophilized low-concentration preparation (Present Invention)

A lyophilized low-concentration HGF preparation can be obtained in the same manner as in Example 8 by using lysine instead of arginine.

Example 20: Preparation of a lyophilized low-concentration preparation (Present Invention)

A lyophilized low-concentration HGF preparation can be obtained in the same manner as in Example 8 by using histidine instead of arginine.

Example 21: Preparation of a lyophilized low-concentration preparation (Present Invention)

A lyophilized low-concentration HGF preparation can be obtained in the same manner as in Example 8 by using glutamine instead of arginine.

Example 22: Preparation of a lyophilized low-concentration preparation (Present Invention)

A lyophilized low-concentration HGF preparation can be obtained in the same manner as in Example 8 by using cysteine instead of arginine.

Example 23: Preparation of a lyophilized low-concentration preparation (Present Invention)

A lyophilized low-concentration HGF preparation can be obtained in the same manner as in Example 8 by using proline instead of arginine.

Example 24: Preparation of a lyophilized low-concentration preparation (Present Invention)

A lyophilized low-concentration HGF preparation can be obtained in the same manner as in Example 8 by using sodium glutamate instead of arginine.

Example 25: Preparation of a lyophilized low-concentration preparation (Present Invention)

A lyophilized low-concentration HGF preparation can be obtained in the same manner as in Example 8 by using sodium aspartate instead of arginine.

Example 26: Preparation of a lyophilized low-concentration preparation (Present Invention)

A lyophilized low-concentration HGF preparation can be obtained in the same manner as in Example 8 by using glycine instead of arginine.

Example 27: Preparation of a lyophilized low-concentration preparation (Present Invention)

A lyophilized low-concentration HGF preparation can be obtained in the same manner as in Example 8 by using a charging amount of 5 mL each instead of 2 mL.

Example 28: Preparation of a lyophilized low concentration preparation (Present Invention)

Sodium dextran sulfate and HGF are dissolved at concentrations of 50 mg/mL and 1 mg/mL, respectively, in 10 mM sodium phosphate buffer (pH 6.5) containing 140 mM sodium chloride and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution is adjusted, the solution is aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation can be obtained by lyophilizing the solution according to the same conditions as in Example 1.

Test Example 1: Evaluation of solubility of HGF

(1) Method for evaluating solubility of HGF

HGF was weighed in a polypropylene tube and added with 10 mM sodium phosphate buffer containing sodium chloride and a stabilizing agent at various concentrations and 0.01% Polysorbate 80. The tube was immediately maintained at a constant temperature to dissolve HGF. Immediately after the dissolution, the solution was subjected to centrifugation (15,000 rpm, 10 minutes, constant temperature) to completely separate the saturated HGF solution and undissolved HGF. The supernatant was sampled and filtered through a low protein adsorptive filter, Millipore GV (hydrophilic Durapore, 0.22 μ m), and HGF concentration of the resulting saturated solution was quantified by HPLC (gel filtration method) to determine solubility of HGF at saturation.

Conditions for HPLC analysis

Column: TOSOH TSK G-3000SWXL (ϕ 0.78 x 30 cm)

Flow rate: 0.3 mL/min

Detection wavelength: OD 280 nm

Temperature: 30°C

Carrier: 0.3 M NaCl, 50 mM sodium phosphate, 0.1% SDS, pH 7.5

Application: 50 μ l

Retention time of HGF: 24.0 min

(2) Influence of pH on solubility of HGF

Solutions of different pH were prepared by using 10 mM sodium phosphate buffer containing 140 mM sodium chloride and 0.01% Polysorbate 80. The solubility of HGF was examined at 4°C and 20°C by the method of (1). The results are shown in Table 2. The solubility of HGF gradually increased with the decrease of pH. Marked improvement of the solubility was found at pH 5.0 or lower. Further, increase of solubility with elevation of temperature was observed in every sample.

Table 2

	20°C	4°C
pH 7.5	0.8	0.4
pH 7.0	1.8	1.0
pH 6.0	2.3	1.3
pH 5.0	5.9	4.2

(Solubility of HGF is shown in mg/mL)

(3) Influences of sodium chloride concentration on solubility of HGF

10 mM Sodium phosphate buffer solutions (pH 7.5) containing sodium chloride at various concentrations and 0.01% Polysorbate 80 were prepared. The solubility of HGF was examined at 4°C and 20°C by the method of (1). The results are shown in Table 3. Remarkable increase of solubility of HGF was observed with the increase of sodium chloride concentration. Further, increase of solubility with elevation of temperature was observed in every sample.

Table 3

	20°C	4°C
Not added	0.3	0.1
+ 140 mM NaCl	0.8	0.4
+ 230 mM NaCl	3.2	1.4
+ 300 mM NaCl	8.5	4.0
+ 900 mM NaCl	> 190	-

(Solubility of HGF is indicated in mg/mL)

(4) Influence of various stabilizing agents on solubility of HGF

Influence of various additives for pharmaceutical preparations on solubility of HGF was examined. HGF was dissolved at a concentration of 1 mg/mL in 10 mM sodium phosphate buffer solutions (pH 6.8-7.5) containing additives at various concentrations, 140 mM sodium chloride and 0.01% Polysorbate 80 to obtain aqueous HGF solutions. An amount of 200 μ L of each aqueous solution was introduced into each well of a 96-well microtiter plate and stored at 4°C for 48 hours. Then, turbidity of each aqueous HGF solution was determined by measuring OD at 450 nm using a plate reader. The turbidity of the solution increased with the decrease of the solubility of HGF which resulted in aggregation and precipitation of HGF.

Influence on HGF solubility was evaluated for additives including 20 kinds of L-amino acids (arginine, lysine, histidine, serine, threonine, asparagine, glutamine, sodium aspartate, sodium glutamate, cysteine, glycine, proline, alanine, isoleucine, leucine, methionine, phenylalanine, tyrosine, tryptophan, valine), 7 kinds of saccharides (mannitol, fructose, trehalose, glucose, sorbitol, sucrose, lactose), 3 kinds of polymers (dextran sulfate, dextran, PEG), 3 kinds of proteins (human serum albumin, acidic gelatin, basic gelatin) and 3 kinds of surface active agents (Polysorbate 80, Polysorbate 20, HCO-40, HCO-60). A stabilization effect to maintain the solubility of HGF was observed in the substances listed below.

(i) Amino acids: arginine, lysine, histidine, sodium glutamate, sodium aspartate, glutamine, cysteine, proline (the effect was confirmed at 0.05 M)
(ii) Polysaccharides: dextran sulfate (the effect was confirmed at 0.1%)

By using the amino acids that gave remarkable effects, solutions were prepared in 10 mM sodium phosphate buffer solutions (pH 7.0) which contained 140 mM sodium chloride, 0.01% Polysorbate 80, and each of the amino acids at a variety of concentrations. The solubility of HGF was examined at 4°C by the method of (1). The results are shown in Table 4.

Table 4

		Saturation solubility	Osmotic pressure ratio
No additive		1.0	1.0
+ L-Arg	50 mM	7.3	1.3
+ L-Lys	50 mM	4.5	1.3
+ L-His	50 mM	3.2	1.2
+ L-GluNa	50 mM	2.2	1.3
+ L-Arg	100 mM	> 10	1.6
+ L-Lys	100 mM	> 10	1.6
+ L-His	100 mM	4.8	1.4
+ L-GluNa	100 mM	3.2	1.6

(Solubility of HGF is shown in mg/mL)

Test Example 2: Properties of aqueous HGF solutions before and after lyophilization

To observe any change in physical stability of HGF during the lyophilization process, an aqueous HGF solution before lyophilization and an aqueous HGF solution obtained by redissolving the lyophilized preparation in purified water without any further treatment were stored at 4°C for 24 hours, and a property (cloudiness) of the solutions after dissolution was visually observed. The time required for redissolution of the lyophilized preparation and the osmotic pressure ratio were also evaluated. The results are shown in Table 5.

When the lyophilized preparations of Examples 1 and 22 were redissolved and stored at 4°C for 24 hours, the solutions became cloudy. The preparations of other examples were found to be stable as to the above property.

Table 5

Preparation	Aqueous solution before lyophilization	Aqueous solution after redissolution	Osmotic pressure ratio
Example 1	Cloudy	Instantly soluble, cloudy	1.0
Example 4	Clear	Instantly soluble, clear	2.0
Example 5	Clear	Hardly soluble, clear	4.0
Example 6	Clear	Hardly soluble, clear	3.9
Example 8	Clear	Instantly soluble, clear	1.5
Example 19	Clear	Instantly soluble, clear	1.6
Example 20	Clear	Instantly soluble, clear	1.4
Example 21	Clear	Instantly soluble, clear	1.3
Example 22	Clear	Hardly soluble, cloudy	1.7
Example 23	Clear	Instantly soluble, clear	1.3
Example 24	Clear	Instantly soluble, clear	1.5
Example 25	Clear	Instantly soluble, clear	1.5
Example 26	Clear	Instantly soluble, clear	1.3
Example 28	Clear	Instantly soluble, clear	1.3

Test Example 3: Properties of lyophilized preparation after dissolution

Time required for redissolution and a solution property (cloudiness) after redissolution of the lyophilized preparations obtained in the examples were evaluated immediately after lyophilization and after storage at 25°C, 40°C and 50°C for 1 month. The lyophilized preparations were dissolved in purified water and the property was evaluated at room temperature. The results are shown in Table 6.

Among those stored at 25°C, the solution of the preparation of Example 22 became cloudy immediately after redissolution of the lyophilized preparation, whilst the preparations of the other examples were found to be stable as to the property. Further, under storage at 40°C and 50°C, the solutions of the preparations of Examples 1, 22, 23, 24 and 25 became cloudy immediately after dissolution. However, the preparations of the other examples were found to be stable as to the above property.

Table 6

Preparation	Preparation after storage for 1 month		
	25°C	40°C	50°C
Example 1	Instantly soluble, clear	Instantly soluble, cloudy	Instantly soluble, cloudy
Example 4	Instantly soluble, clear	Instantly soluble, clear	Instantly soluble, clear
Example 5	Hardly soluble, clear	Hardly soluble, clear	Hardly soluble, clear
Example 6	Hardly soluble, clear	Hardly soluble, clear	Hardly soluble, clear
Example 8	Instantly soluble, clear	Instantly soluble, clear	Instantly soluble, clear
Example 19	Instantly soluble, clear	Instantly soluble, clear	Instantly soluble, clear
Example 20	Instantly soluble, clear	Instantly soluble, clear	Instantly soluble, clear
Example 21	Instantly soluble, clear	Instantly soluble, clear	Instantly soluble, clear
Example 22	Hardly soluble, cloudy	Hardly soluble, cloudy	Hardly soluble, cloudy
Example 23	Instantly soluble, clear	Instantly soluble, cloudy	Instantly soluble, cloudy
Example 24	Instantly soluble, clear	Instantly soluble, cloudy	Instantly soluble, cloudy
Example 25	Instantly soluble, clear	Instantly soluble, cloudy	Instantly soluble, cloudy
Example 26	Instantly soluble, clear	Instantly soluble, clear	Instantly soluble, clear
Example 28	Instantly soluble, clear	Instantly soluble, clear	Instantly soluble, clear

Test Example 4: Change in a aggregate content in a lyophilized preparation

The ratio of a aggregate content and an HGF content in the lyophilized preparations obtained in the examples were compared immediately after lyophilization (initial value) and after storage at 25°C, 40°C and 50°C for 1 month. The method of Test Example 1 (1) (gel filtration method) was applied. The results are shown in Tables 7 and 8.

Retention time of aggregates: 20.4 min, 21.8 min
Retention time of HGF: 24.0 min

With the elevation of the storage temperature, an increasing tendency of aggregate formation was observed. However, the lyophilized preparations of Examples 8, 19 and 20, in particular, gave extremely low aggregate formation and they were found to be physicochemically stable. It was thus concluded that addition of arginine, lysine and histidine kept aggregate formation at a low level even after high-temperature storage (aggregate formation rate: about 3% or lower at 40°C and about 5-9% or lower at 50°C), thereby improved storage stability. A similar test was performed as a comparative example by using a lyophilized preparation of Example 1 which was obtained according to the same method using the same ingredients except that arginine was not contained. As a result, marked increase in aggregate formation was observed with the elevation of the storage temperature.

Further, as shown in Table 8, it was demonstrated that decrease of the HGF concentration (lower than 5 mg/mL) accelerated aggregate formation, which resulted in lower storage stability. It was found that, when glycine or alanine was used as a stabilizing agent of the lyophilized preparation as described in Japanese Patent Unexamined Publication No. 9-25411, aggregate formation was accelerated and storage stability was lowered in the lyophilized low-concentration HGF preparations (Examples 5, 6 and 26, 1 mg/mL) as compared to the lyophilized high-concentration HGF preparations (Examples 5 and 6 in Japanese Patent Unexamined Publication No. 9-25411, 20 mg/mL).

Whilst it was demonstrated that, in the lyophilized preparations of Examples 8, 19 and 20 wherein arginine, lysine or histidine was used as a stabilizing agent of the lyophilized preparation, aggregate formation was markedly suppressed and thus

storage stability was improved even in the lyophilized low-concentration HGF preparations (1 mg/mL).

Table 7

Preparation	Preparation after storage for 1 month			
	Initial value	25°C	40°C	50°C
Example 1	0.48	5.50	24.27	40.63
Example 4	0.35	0.48	3.80	11.24
Example 5	0.31	0.69	4.40	9.58
Example 6	0.30	0.54	3.20	9.53
Example 8	0.30	0.11	0.18	0.60
Example 19	0.31	0.16	1.74	4.48
Example 20	0.31	0.18	0.28	0.88
Example 21	0.31	0.54	2.77	16.89
Example 22	-	-	-	-
Example 23	0.32	0.31	3.24	11.24
Example 24	0.32	2.19	4.90	6.39
Example 25	0.34	1.11	4.80	7.73
Example 26	0.36	0.33	6.21	22.66
Example 28	2.74	3.48	13.30	32.87
Example 1*	1.07			6.17
Example 5*	0.92			4.09
Example 6*	0.93			2.90
Example 9*	1.78			14.01

* Quoted from Tables 4 and 6 in Japanese Patent Unexamined Publication No. 9-25241

Table 8

Preparation		Aggregate content/HGF content of lyophilized preparation stored at 50°C for 1 month
[Preparation containing no amino acid]		
Example 1	(HGF at 1 mg/mL)	40.63
Example 4	(HGF at 1 mg/mL)	11.24
Example 9*	(HGF at 10 mg/mL)	14.01
Example 1*	(HGF at 20 mg/mL)	6.17
[Preparation containing glycine]		
Example 5	(HGF at 1 mg/mL)	9.58
Example 5*	(HGF at 20 mg/mL)	4.09
[Preparation containing alanine]		
Example 6	(HGF at 1 mg/mL)	9.53
Example 6*	(HGF at 20 mg/mL)	2.90

* Quoted from Tables 4 and 6 in Japanese Patent Unexamined Publication No. 9-25241

Test Example 5: Change in aggregate content in lyophilized preparation - influence of pH on aggregate formation

The ratio of the aggregate content and the HGF content in the lyophilized preparations having various pH prepared in Examples 8, 13, 14, 16 and 17 was determined immediately after lyophilization (initial value) and after storage at 50°C for 1 month, 2 months and 3 months. The method of Test Example 1 (1) (gel filtration method) was used. The results are shown in Table 9.

Retention time of aggregate: 20.4 min, 21.8 min
Retention time of HGF: 24.0 min

In the lyophilized preparations of Examples 16 and 17 having pH of 7.0 and 7.2, aggregate formation was increased with time during the storage at 50°C. Whilst in the preparations of Example 8, 13 and 14, which had pH of 6.5 or lower, aggregate

formation was kept at a low level. It was thus concluded that the stability was improved under weakly acidic pH.

Table 9

Preparation	Initial value	Stored at 50°C		
		Stored for 1 month	Stored for 2 months	Stored for 3 months
Example 14 (pH 5.5)	0.48	0.38	0.56	0.76
Example 13 (pH 6.0)	0.35	0.91	0.51	1.31
Example 8 (pH 6.5)	0.31	1.40	1.58	1.28
Example 17 (pH 7.0)	0.30	1.26	2.16	2.96
Example 16 (pH 7.2)	0.30	5.64	7.63	12.71

Test Example 6: Change in biological activity (specific activity) of lyophilized preparation

The lyophilized preparations prepared in Examples 1 and 8 were stored at 25°C or 50°C for 2 months or at 10°C or 25°C for 1.5 years. The biological activities of aqueous solutions obtained by redissolving the lyophilized preparations were determined by the method for determining biological activity shown below. The results are shown in Table 10.

Method for determining biological activity

A human hepatic cell strain PLC/PRF/5 was cultured until the logarithmic growth phase, and the cell survival rate was observed. Then, a cell solution was prepared at a density of 0.7×10^5 cells/mL. An amount of 100 μ L of the cell solution was put into each well of a 96-well assay plate added beforehand with an HGF sample

or a standard sample so that the cell count was 0.7×10^4 cells/well (n = 4). After pre-incubation at 37°C for 20 hours in a 5% carbon dioxide incubator, [^{3}H-thymidine] was added and the culture was further continued for 6 hours. After completion of the culture, cells were collected by using a Beta Plate System (Pharmacia) and the amount of [^{3}H] taken up into the cells was measured. The measurement results were verified by the parallel line test. The titer (%) was obtained by dividing the specific activity of the HGF sample by the specific activity of the standard.

As for the lyophilized preparation of Example 8 added with arginine, the biological activity was almost unchanged even after the high-temperature storage and the preparation was stable as for the biological activity. Further, a similar test was performed as a comparative example by using a lyophilized preparation obtained by the same method with the same ingredients as in Example 1 except that arginine was not contained. As a result, a marked decrease in biological activity was observed with the elevation of the storage temperature.

Table 10

	Stored for 2 months		Stored for 1.5 years	
	25°C	50°C	10°C	25°C
Example 1	64.5%	10.2%	-	-
Example 8	87.1%	71.0%	72.6%	66.1%

Industrial Applicability

The lyophilized preparation of the present invention can be used to prepare a clinically useful aqueous solution containing HGF at a low concentration, and said preparation is almost free from aggregate formation during lyophilization and storage after the lyophilization and thus has excellent stability. Further, said preparation is characterized by favorable cake forming property during lyophilization and excellent re-solubility. In addition, said preparation can also be made into a preparation having a pH and an osmotic pressure ratio desirable as an injection.

What is claimed is:

1. A lyophilized preparation comprising a hepatocyte growth factor, a stabilizing agent for preventing formation of a aggregate of the hepatocyte growth factor, sodium chloride, and a buffering agent, which is prepared from an aqueous solution containing the hepatocyte growth factor at a concentration lower than 5 mg/mL.

2. A lyophilized preparation comprising a hepatocyte growth factor, a stabilizing agent for preventing formation of a aggregate of the hepatocyte growth factor, sodium chloride, and a buffering agent, which is used for preparing an aqueous solution containing the hepatocyte growth factor at a concentration lower than 5 mg/mL by redissolution.

3. A lyophilized preparation comprising a hepatocyte growth factor, a stabilizing agent for preventing formation of a aggregate of the hepatocyte growth factor, sodium chloride, and a buffering agent, which is prepared from an aqueous solution containing the hepatocyte growth factor at a concentration lower than 5 mg/mL and used for preparing an aqueous solution containing the hepatocyte growth factor at a concentration lower than 5 mg/mL by redissolution.

4. The lyophilized preparation according to any one of claims 1 to 3, wherein the stabilizing agent is selected from the group consisting of arginine, lysine, histidine, glutamine, proline, glutamic acid, aspartic acid, sulfated polysaccharides, and a pharmacologically acceptable salt thereof.

5. The lyophilized preparation according to any one of claims 1 to 3, wherein the stabilizing agent is selected from the group consisting of arginine, lysine, histidine, glutamic acid, aspartic acid, and a pharmacologically acceptable salt thereof.

6. The lyophilized preparation according to any one of claims 1 to 3, wherein the stabilizing agent is selected from the group consisting of arginine, lysine, histidine, and a pharmacologically acceptable salt thereof.

7. The lyophilized preparation according to any one of claims 1 to 6, wherein the buffering agent is a phosphoric acid salt.

8. The lyophilized preparation according to any one of claims 1 to 7, wherein the aqueous solution before lyophilization has a pH and an osmotic pressure ratio desirable as an injection.

9. The lyophilized preparation according to any one of claims 1 to 7, wherein the aqueous solution obtained after redissolution has a pH and an osmotic pressure ratio desirable as an injection.

10. The lyophilized preparation according to claim 8 or 9, wherein a pH of the aqueous solution before lyophilization is in the range of 5 to 6.5.

11. The lyophilized preparation according to claim 8 or 9, wherein a pH of the aqueous solution obtained after redissolution is in the range of 5 to 6.5.

12. The lyophilized preparation according to any one of claims 1 to 11, which further contains a surface active agent.

13. The lyophilized preparation according to claim 12, wherein the surface active agent is a nonionic surface active agent.

14. The lyophilized preparation according to claim 13, wherein the nonionic surface active agent is a polyoxyethylene ether surface active agent.

15. The lyophilized preparation according to any one of claims 1 to 14, which is prepared in a vial or an ampoule.

16. The lyophilized preparation according to any one of claims 1 to 15, which contains the stabilizing agent in an amount sufficient to prevent HGF aggregate formation during lyophilization and/or storage after the lyophilization.

17. A stabilizing agent for HGF used to lyophilize an aqueous solution containing HGF at a concentration lower than 5 mg/mL, which is selected from the group consisting of arginine, lysine, histidine, glutamine, proline, glutamic acid, aspartic acid, sulfated polysaccharides, and a pharmacologically acceptable salt thereof.

18. The stabilizing agent according to claim 17, which can prevent HGF aggregate formation during lyophilization and/or storage after the lyophilization.

19. The stabilizing agent according to claim 17, which is used in an amount sufficient to prevent HGF aggregate formation during lyophilization and/or storage after the lyophilization.

20. The stabilizing agent according to any one of claims 17 to 19, which is selected from the group consisting of arginine, lysine, histidine, glutamic acid, aspartic acid, and a pharmacologically acceptable salt thereof.

21. The stabilizing agent according to any one of claims 17 to 19, which is selected from the group consisting of arginine, lysine, and a pharmacologically

acceptable salt thereof.

Docket #: p21749.dc1.doc

Declaration and Power of Attorney for Utility or Design Patent Application
特許出願宣言書

Japanese Language Declaration

私は、下欄に氏名を記載した発明者として、以下のとおり宣言する：

私の住所、郵便の宛先および国籍は、下欄に氏名に続いて記載したとおりであり、

名称の発明に関し、請求の範囲に記載した特許を求める主題の本来の、最初にして唯一の発明者である（一人の氏名のみが下欄に記載されている場合）か、もしくは本来の、最初にして共同の発明者である（複数の氏名が下欄に記載されている場合）と信じ、

上記発明の明細書（下記の欄で X 印がついていない場合は、本書に添付）は、

☐ ____年____月____日に提出され、米国出願番号 ____ とし、（該当する場合）

☐ ____年____月____日に訂正されました。又は、

☐ 特許協定条約国際出願番号 ____ とし、（該当する場合）____年____月____日に訂正されました。

私は、前記のとおり補正した請求の範囲を含む前記明細書の内容を検討し、理解したことを陳述する。

私は、連邦規則法典第 37 編第 1 条 56 項に定義されているとおり特許資格の有無について重要な情報を開示すべき義務があることを認めます。

私は、合衆国法典第 35 部第 119 条 (a-d) 項又は第 365 条(b)項に基づく、下記の外国特許出願又は発明者証出願、或いは第 365 条(a)項に基づく、少なくても米国以外の 1 カ国を指名した PCT 国際出願の外国優先権を主張し、更に優先権の主張に係わる基礎出願の出願日前の出願日を有する外国特許出願、又は発明者証出願或るいは PCT 国際出願を以下に“なし”の箱に印をつけることにより明記する：

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name:

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
Lyophilized HGF Preparation

the specification of which is attached hereto unless the following box is checked:

☒ was filed on **31/May/00** as United States Application Number **09/926,661** and was amended on **29/Nov/01** (if applicable) or,

PCT International Application Number **PCT/JP00/03506** and was amended on ____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority under Title 35, United States Code §119(a-d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States, listed below. I have also identified below, by checking the "No" box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed:

Prior foreign applications
先の外国出願

(Number) (番号)	(Country) (国名)	(Day/Month/Year Filed) (出願の年月日)	Priority claimed 優先権の主張 Yes あり	No なし
11-161769	**Japan**	**31/May/99**	☒	☐
			☐	☐

☐ その他の外国特許出願番号は別紙の追補優先権欄にて記載する。

☐ Additional foreign application numbers are listed on a supplemental priority sheet attached hereto.

Japanese Language Utility or Design Patent Application Declaration

私は、合衆国法典第 35 部第 119 条(e)項に基づく、下記の合衆国仮特許出願の利益を主張する。

I hereby claim the benefit under Title 35, United States Code §119 (e) of any United States provisional application(s) listed below.

(Application No.)
（出願番号）

(Day/Month/Year Filed)
（出願の年月日）

(Application No.)
（出願番号）

(Day/Month/Year Filed)
（出願の年月日）

(Application No.)
（出願番号）

(Day/Month/Year Filed)
（出願の年月日）

☐ その他の合衆国仮特許出願番号は別紙の追補優先権欄にて記載する。

☐ Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

私は、合衆国法典第 35 部第 120 条に基づく下記の合衆国特許出願、又は第 365 条(c)項に基づく合衆国を指名した PCT 国際出願の利益を主張し、本願の請求の範囲各項に記載の主題が合衆国法典第 35 部第 112 条第 1 項規定の態様で、先の合衆国特許出願又は PCT 国際出願に開示されていない限度において、先の出願の出願日と本願の国内出願日又は PCT 国際出願日の間に有効となった連邦規則法典第 37 部第 1 章第 56 条に記載の特許要件に所要の情報を開示すべき義務を有することを認める。

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s), or §365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

(Application No.)
（出願番号）

(Day/Month/Year Filed)
（出願の年月日）

(Status)
（現況）
（特許済み、係属中　放棄済み）
(patented, pending, abandoned)

(Application No.)
（出願番号）

(Day/Month/Year Filed)
（出願の年月日）

(Status)
（現況）
（特許済み、係属中　放棄済み）
(patented, pending, abandoned)

☐ その他の合衆国又は国際特許出願番号は別紙の追補優先権欄にて記載する。

☐ Additional U.S. or international application numbers are listed on a supplemental priority sheet attached hereto.

私は、ここに自己のに知識に基づいて行った陳述が全て真実であり、自己の有する情報および信ずるところに従って行った陳述が真実であると信じ、さらに故意に虚偽の陳述等を行った場合、合衆国法典第 18 部第 1001 条により、罰金もしくは禁　に処せられるか、またはこれらの刑が併科され、またかかる故意による虚偽による陳述が本願ないし本願に対して付与される特許の有効性を損なうことがあることを認識して、以上の陳述を行ったことを宣言する。

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

私、下記署名者は、ここに記載の米国弁護士または代理人に本出願に関し特許商標庁にて取られるいかなる行為に関して、同米国弁護士又は代理人が私に直接連絡なしに私の外国弁護士或るいは法人代表者からの指示を受け取り、それに従うようここに委任する。この指示を出す者が変更の場合には、ここに記載の米国弁護士又は代理人にその旨通知される。

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from either his foreign patent agent or corporate representative, if any, as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

Docket #: p21749.dc1.doc

Japanese Language Utility or Design Patent Application Declaration

委任状： 私は、下記発明者として、下記に明記された顧客番号を伴う以下の弁護士又は、代理人をここに選任し、本願の手続きを遂行すること並びにこれに関する一切の行為を特許商標庁に対して行うことを委任する。そして全ての通信はこの顧客番号宛に発送される。

顧客番号 7055

現在委任された弁護士は下記の通りである。

Neil F. Greenblum	Reg. No. 28,394
Bruce H. Bernstein	Reg. No. 29,027
James L. Rowland	Reg. No. 32,674
Arnold Turk	Reg. No. 33,094

POWER OF ATTORNEY: As a named inventor, I hereby appoint the attorney(s) and/or agent(s) associated with the Customer Number provided below to prosecute this application and transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number:

CUSTOMER NUMBER 7055

The appointed attorneys presently include:

Stephen M. Roylance	Reg. No. 31,296
William E. Lyddane	Reg. No. 41,568
William Pieprz	Reg. No. 33,630
Leslie J. Paperner	Reg. No. 33,329

Address: **GREENBLUM & BERNSTEIN, P.L.C.**
1941 Roland Clarke Place
Reston, VA 20191

直接電話連絡先： Direct Telephone Calls to:

GREENBLUM & BERNSTEIN, P.L.C.
(703) 716-1191

唯一のまたは第一の発明者の氏名	Full name of sole or first inventor Masatoshi CHIBA
同発明者の署名 日付	Inventor's signature Date Masatoshi Chiba February 4, 2002
住所	Residence Ibaraki, JAPAN
国籍	Citizenship Japan
郵便の宛先	Post Office Address c/o Mitsubishi Chemical Corporation, Kashima Plant 14, Sunayama, Hazaki-machi, Kashima-gun, Ibaraki 314-0255, Japan
第二の共同発明者の氏名（該当する場合）	Full name of second joint inventor, if any
同第二共同発明者の署名 日付	Second Inventor's signature Date
住所	Residence
国籍	Citizenship
郵便の宛先	Post Office Address

（第三またはそれ以降の共同発明者に対しても同様な情報および署名を提供すること。）

(Supply similar information and signature for third and subsequent joint inventors.)